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14. ABSTRACT

The most significant accomplishment during the current funding period is the demonstration that blocking specific interactions between microRNAs and the 3' noncoding portion of the fragile X (*FMR1*) gene leads to increased *FMR1* protein production. Reduced levels of FMRP are responsible for the leading heritable form of intellectual disability, fragile X syndrome. The development of a means for increasing FMRP levels raises the expectation of a therapeutic approach for correcting all of the clinical domains of fragile X syndrome, including epileptiform activity observed for both those with FXS and carriers of smaller CGG-repeat expansions. The short-term outcomes of the proposed research are therapeutic targets for upregulation of FMRP, and the demonstration that such upregulation ameliorates the dysregulation caused by FMRP deficiency. Low FMRP is also found in some patients with autism and other psychiatric problems without FXS, so the long-term therapeutic implications of this research go far beyond FXS.

15. SUBJECT TERMS
FMRP induction, fragile X syndrome, autism, post-traumatic stress disorder (PTSD)

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INTRODUCTION

Fragile X syndrome (FXS) is the most common heritable form of intellectual disability, the most common single-gene form of autism, and a relatively common cause of epilepsy. The syndrome is caused by partial or complete silencing of the fragile X (*FMR1*) gene when a CGG-repeat element in the gene expands to more than 200 repeats, leading in turn to loss of the *FMR1* protein (FMRP). The protein is important for brain development, and its loss is accompanied by both intellectual and behavioral disability. Accordingly, the central objective of the proposed research is identification of therapeutic agents that stimulate production of FMRP from residual *FMR1* messenger (m)RNA in neurons, thereby reversing the effects of decreased gene activity. Our approach is to block the interactions between microRNAs and the 3' non-coding portion of the *FMR1* message; such interactions are known to decrease protein production, so blocking such interactions should raise FMRP levels. Increasing the expression of FMRP holds the potential to correct ALL of the clinical domains of fragile X syndrome, including epilepsy-like activity observed for both those with FXS and carriers of smaller CGGrepeat expansions. Finally, posttraumatic stress disorder (PTSD) has been described in fragile X syndrome and in premutation carriers. Thus, the proposed studies may lead to treatments that reduce the PTSD risk as well, an issue of importance for the military personnel. Since the prevalence of fragile X syndrome is approximately 1 in 3,000 to 4,000 in the general population, nearly two-thousand children of service personnel are likely to have fragile X syndrome, with a much larger number (~7,500) of active military personnel being carriers of an expanded (premutation) form of the *FMR1* gene.

BODY

Task 1 (inclusive of sub-tasks 1a, b, c). *Identification of cis-elements for translational up-regulation within the FMR1 mRNA non-coding regions (timeframe, < 18 months; Hagerman).*

Overview of progress: In our original proposal under this task, we had described the development of a surrogate (reporter) mRNA that contains all elements of the *FMR1* mRNA sequence except for the substitution of a reporter protein (eGFP) coding region for the FMRP coding region. This reporter plasmid was to be transfected into control and expanded-CGG-repeat–bearing fibroblast lines. The primary reporter plasmid (no modifications to *cis*-elements) has been constructed, and the construct has been transfected into control and expanded-CGG-repeat–bearing human fibroblast lines. The transfection efficiency has been ~50%, as determined by *in situ* eGFP when examined under epifluorescence.

Although our transfection protocol has been optimized for fibroblasts, the same protocol produces significant toxicity when applied to neurons derived from either human iPSCs or the mouse premutation model. We have therefore piloted a second method that uses the cell penetrating peptide, penetratin (review: Dupont et al., 2011). Four-day-in-vitro (DIV) mouse neurons were exposed to penetratin conjugated to Alexa 488 fluorophore for 24 hours. Optimization of the peptide concentration and exposure protocol yielded over 40% of the neurons showed strong internalization of the dye into the cytosol.

During the course of these experiments, we realized that an important approach to the same problem would be to use the native protein (FMRP) as a "reporter" to block various targets along the 3′UTR of the *FMR1* message. In other words, as a complement to the studies with the exogenous (eGFP) reporter, we could use synthetic RNA analogues (e.g., BNAs; Prakash, 2011) to block the target sequences of one or more microRNAs along the *FMR1* 3′UTR. This approach does not suffer the adverse effects (partial transfection, transfection toxicity) associated with the eGFP reporter.

Through the target blocking experiments, we have completed much of Task 1. In the first component of these experiments, we first determined the profile of all micro(mi)RNAs in the cell types under study, the idea being that it would do no good to block a site for which there was no appreciable miRNA. For the next component, we analyzed (*in silico*) the entire 2.2kb of the 3′UTR to determine which sequence elements fit criteria for miRNA binding (including strength of binding; ~100 targets). The combination of miRNA concentrations x target binding strength yielded a predicted set of targets for blocking (**Figure 1**).

On the basis of the information in **Figure 1**, we designed a set of BNAs to act as target blockers and measured the change of endogenous FMRP following addition of the BNAs, singly or in combination (**Figure 2**). The best target blocker was one that prevented the binding of miR19b, resulting in an average increase of ~70% in the level of FMRP. However, an unexpected observation was that part of this increase is likely due to

protection of the mRNA from degradation, presumably through the action of miR19b in an RNAi capacity. These experiments represent a substantial completion of Task 1; however, additional experiments would be warranted to determine to what extent greater coverage of miRNA binding sites (simultaneous binding of multiple target blockers) further increase FMRP levels through a combination of increased mRNA stability and direct translational upregulation.

Task 2. ***Small molecule screening of a specific (CNS-set ChemBridge) library of ~50,000 small molecules for candidates capable of increasing FMRP and eGFP levels in transfected fibroblasts (timeframe, <18 months; Hagerman).***

Overview of progress: We have obtained ~18,000 drug-like small molecules, each with increased probability of crossing the blood-brain-barrier, from ChemBridge (ChemBridge Corporation; San Diego, CA). The smaller number of compounds reflected the increased price of each compound for the formulation required for our experiments. Our original approach was to use each drug (in DMSO) plated into 384-well culture plates, followed by the addition of (eGFP transfected) fibroblasts. Levels of eGFP would be measured via plate-based fluorometry following various time intervals. Positive candidates would be those that increase eGFP fluorescence. As originally envisioned under *Subtask 2a*, small-molecule agonists that are found to increase the expression of eGFP would be subsequently validated by Western for FMRP.

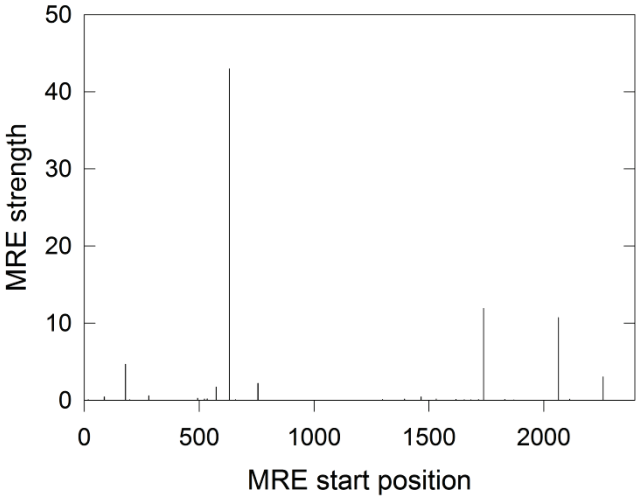


Figure 1. Graph of the relative "strength" of a predicted MRE based on the relative abundance of the targeting miRNAs and the predicted strengths of the miRNA-MRE interactions. The four highest peaks are, from left to right, MREs at nt 179 of the 3′UTR (miR19a,b), nt 631 (miR221, 222), nt 1738 (miR25, 92a), and nt 2064 (miR23a,b).

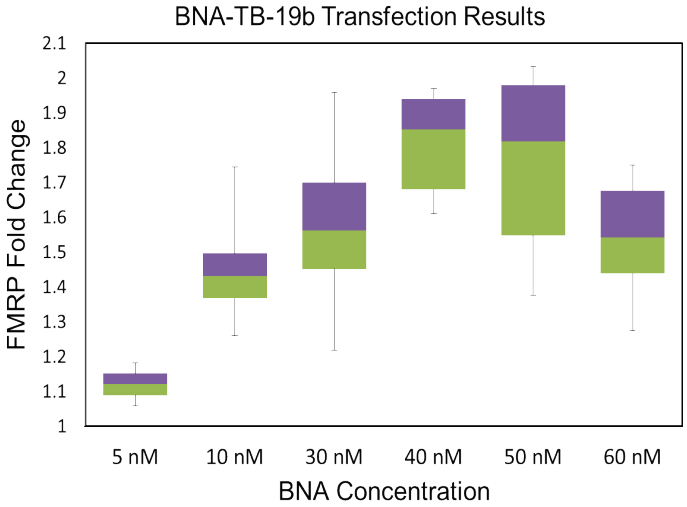
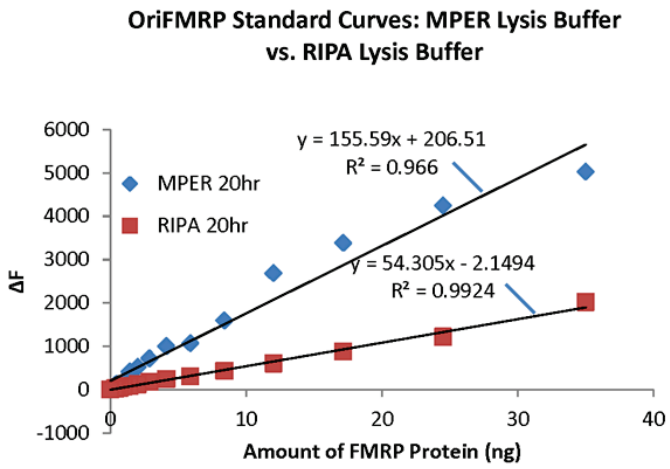


Figure 2. Box plot of the increase in FMRP expression in human dermal fibroblasts as a function of the concentration of the target blocker (BNA) for the MRE starting at nt 179 (miR19a, b). At the concentration of maximal effectiveness, the increase in FMRP is nearly two-fold.

However, in the interim, we have found a far faster method for detecting endogenous FMRP levels, a FRET-based approach originally described by Schutzius et al. (2013), and modified by Cisbio (Cisbio US, Bedford, MA). The idea behind this approach is that a protein can be detected if fluorescence donor- and acceptor-labeled antibodies are bound to the same protein. This method is very rapid, has extremely low background, and is very sensitive to low levels of protein. The added bonus is that it would eliminate the need to use an eGFP reporter for a first step and a Western blot analysis for a second step – the assay can be performed on native FMRP in a single step and in a single well for each compound. We have therefore developed the method in our laboratory prior to screening the compounds; features of the approach are displayed in **Figure 3**.



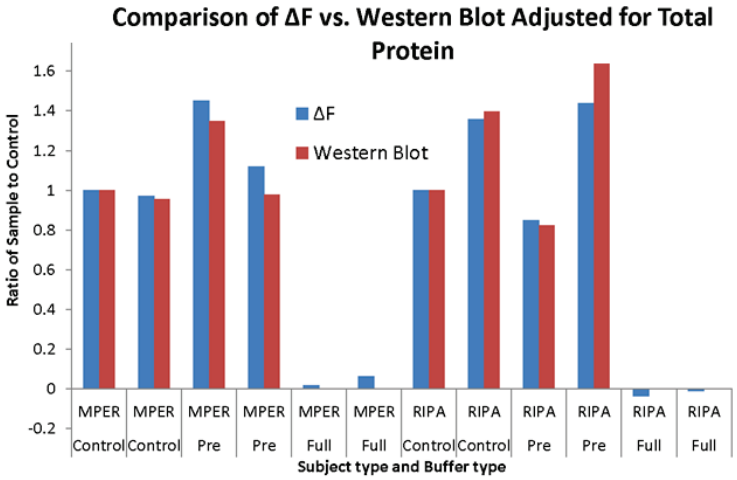


Figure 3. Initial validation studies of FMRP levels using the Casio FRET assay. (Left panel) Standard curve for FMRP (ΔF, change in FRET-derived fluorescence signal using time-resolved fluorescence decay) using two distinct lysis buffer systems (MPER and RIPA). We discovered that RIPA results in substantial quenching. (Right panel) Comparison of ΔF (FRET) versus traditional Western blot approach; the FRET method is far faster and more accurate than Western methods.

Task 3. ***Creation of iPSC-derived and transdifferentiated neurons from fibroblast lines (timeframe < 24 months; Hagerman and Pessah).***

Overview of progress: This task focuses on generating neuronal cell models that express *FMR1* CGG-expansion repeats in the high premutation to full mutation range using two approaches: (1) reprogramming fibroblasts to iPSCs and then promoting neuronal cell differentiation (i.e., establishing neural precursor cell (NPC) lines); and (2) direct transdifferentiation of fibroblast to neuronal cell lineage. Subtask 3a entails the production of at least two iPSC-derived neuronal pairs based on differential activity of normal or expanded X chromosome, 120-200 and >200 range, whereas Subtask 3b was to generate at least five transdifferentiated neuronal pairs based on differential activity of normal or expanded X chromosome. Using these lines, we would perform various morphological and functional analyses using single-cell methods.

Thus far, we have subcloned several fibroblast lines from individuals with *FMR1* alleles in the normal range (<35 CGG repeats), the mid-repeat range (40-100 CGG repeats), the high-repeat range (150-200 CGG repeats), and the FXS range (>200 repeats). Each fibroblast subclonal line is being characterized for stability of the CGG-repeat-expansion size, disruption of lamin A/C surrounding the nucleus, and functional measures of mitochondrial dysfunction; all are obligatory steps to producing iPSCs and the neural precursor cells (NPCs) derived from them.

In this regard, we have determined that CGG-repeat size and FMRP expression levels remain consistently stable under established culture conditions for each fibroblast clonal line. A new and possibly complicating finding, particularly in the premutation ranges, is that *FMR1* mRNA can vary depending on culture conditions, including time out from media changes, days in culture, and passage number. Since mRNA levels are an important determinant of FMRP levels in human patients, this new observation underscores the importance maintaining stringent experimental culture protocols when performing *in vitro* studies. Our preliminary findings indicate detectible alterations in basal and maximal mitochondrial respiratory capacity in fibroblasts expressing *FMR1* CGG expansions. We are currently investigating whether a relationship among these parameters of mitochondrial function, FMRP, and *FMR1* mRNA levels exist and are further exploring their underlying mechanisms.

In year 1, we focused on a comparative analysis of two neuronal culture models of *FMR1*-related disorders. These studies expanded on our initial report identifying morphologic and functional differences between an isogenic pair of iPSC-derived NPCs derived from a female with a mosaic *FMR1* premutation (Liu et al., 2012), a post-mortem human brain (Pretto et al., 2014), and similar abnormalities in the mouse premutation model (Cao et al., 2013; Cao et al., 2012; Kaplan et al., 2012). A detailed analysis has defined in some detail how *FMR1* CGG-repeat size and developmental age in culture influence levels of *FMR1* mRNA, FMRP, and the

patterns of neuronal morphology, including dendritic length and number per neuron (**Figure 4**).

Neuronal *FMR1* mRNA levels are highest in 170 CGG-repeat premutation alleles (pre170) (3-4 fold of wild type (WT)) and intermediate in pre98 (1.5-2 fold of WT) neurons, whereas FMRP levels are lowest in pre170 (~50% of WT) and intermediate in pre98 (75-90% of WT) (**Figure 4**).

These key genotype differences are seen as early as 4 DIV and extend throughout the critical timeframe needed for neuronal cultures to develop the dendritic complexity (**Figure 4**) and synaptic architecture (Chen et al., 2010) sufficient to engage spontaneous synchronous Ca^{2+} oscillations (SCOs; **Figure 5**) and electrical field potentials (Cao et al 2012) across the networks they form. Our data indicates that the onset and progression of both morphological and functional abnormalities are highly dependent on the size of the *FMR1* CGG expansion, with pre98 neurons (**Figure 4B**, upper panel) initially extending longer dendrites with similar number of dendritic branches (**Figure 4B**, lower panel) compared to WT at 4 and 7 DIV. By 21 DIV, both dendritic length and number of branches are significantly less than WT. This finding is sharply contrasted by the pre170 neurons that produce less developed dendritic processes than either pre98 or WT neurons throughout the developmental timeframe measured to date (**Figure 4B**). Such genotype dependent differences in development of neuronal complexity are not limited to *in vitro* cell cultures as they have been detected in Golgi stains of premutation mouse brain sections (Berman et al., 2012; Wenzel et al., 2010), and exhibited altered neuroplasticity in acute hippocampal slice preparations (Hunsaker et al., 2012).

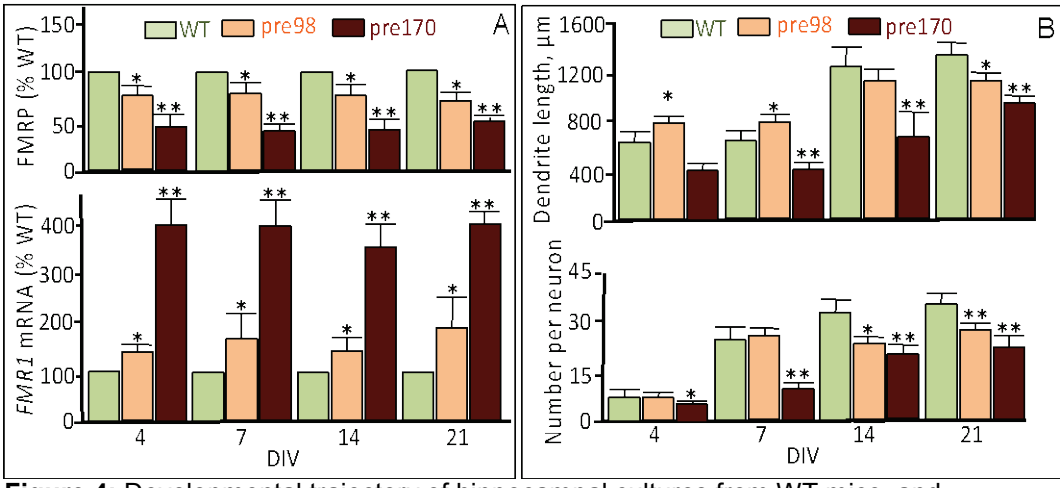


Figure 4: Developmental trajectory of hippocampal cultures from WT mice, and premutation knock-in mice expressing mid (pre98) and high (pre170) CGG repeats. (A) FMRP and *FMR1* mRNA levels relative to WT. (B) Total dendritic length and number of branches (termini) per neuron. Mean ±SEM **p*<0.05; ***p*<0.01.

We directly compared the patterns of SCOs and responses to glutamate challenges elicited by hippocampal neurons cultured from the premutation mouse with iPSC-derived NPCs at critical periods of neurodevelopment. Two observations consistent between the murine and human *in vitro* models significantly differ between *FMR1* premutation and their corresponding normal neurons: (1) significantly higher frequency and clustering of Ca^{2+} oscillations in premutation neurons (**Figure 5A**), and (2) significantly prolonged responses to challenges with glutamate (**Figure 5B**).

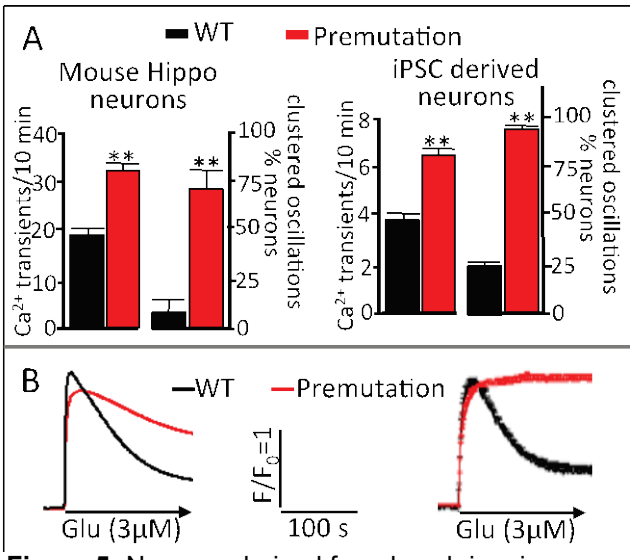


Figure 5: Neurons derived from knock-in mice or human iPSCs exhibit early (7 DIV) abnormal Ca^{2+} dynamics (A) and response to Glu (B).

Recently we provided evidence that abnormal glutamate transport is common both in human (Pretto et al., 2014) and mouse (Cao et al., 2013) *FMR1* premutation. The use of Ca^{2+} selective microelectrodes to record resting free cytoplasmic Ca^{2+} in the presence of tetrodotoxin (TTX ; to block SCOs) indicates that premutation neurons cultured from mice maintain chronically elevated cytoplasmic free Ca^{2+} compared to WT neurons (**Figure 6**). Such alterations in Ca^{2+} dynamics would be expected to not only influence activity dependent dendritic growth patterns, but also influence Ca^{2+}-dependent signaling essential for maintaining normal synaptic connectivity.

To further assess the potential impacts of altered Ca^{2+} dynamics observed with premutation neurons, we investigated whether the levels and patterns of expression of FMRP and *FMR1* mRNA influence those of Cdk5 and ATM, two transcriptional factors critical for orchestrating nuclear and synaptic signals that maintain influence long term neuronal connectivity and neuronal survival, that when disturbed may promote progressive neuronal damage in a feed-forward manner. Consistent with western blot data, immunofluorescence labeling of cultured hippocampal neurons from premutation mice show ~40-50% reduction of FMRP within soma. However, the levels of FMRP within the distal dendrites of premutation neurons are less than 15% than that found in wild type neurons (**Figure 7**).

A new and potentially significant finding linking chronic Ca^{2+} dysregulation, abnormal synaptic connectivity (i.e., altered responses to Glu) and progressive impairments in neuronal health is that high premutation neurons (CGG >170) express much higher levels of both Cdk5 (~2-fold of wild type) and ATM (~1.5-fold) (**Table 1**). Importantly, initial results indicate that P-ATM is upregulated ~5.5-fold in high premutation compared to wild type (**Table 1**).

Table 1. Elevated signaling proteins through chronic calcium dysregulation

Cdk5/β actine (a.u)		ATM/β actine (a.u)		P-ATM Ser1981/β actine (a.u)	
Wt	High cgg	Wt	High cgg	Wt	High cgg
1.095	2.023	0.107	0.148	0.147	0.808

Taken together these are the first results to demonstrate a molecular signaling mechanism that involves chronic activation of two transcriptional factors commitment with an abnormal Ca^{2+} dynamics and distribution of FMRP in high-range premutation neurons. Upregulation and activation of Cdk5 and ATM, possibly the consequence of chronically elevated neuronal Ca^{2+}, can produce feed-forward signaling that alters not only the integrity of nuclear DNA repair but also alteration in synaptic connectivity.

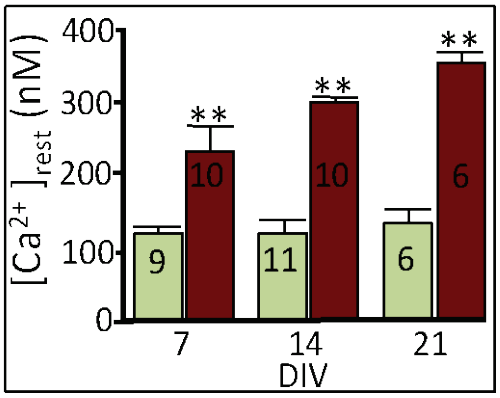


Figure 6: Chronically elevated resting cytoplasmic Ca^{2+} ($[Ca^{2+}]_{rest}$) in pre170 neurons augments with DIV.

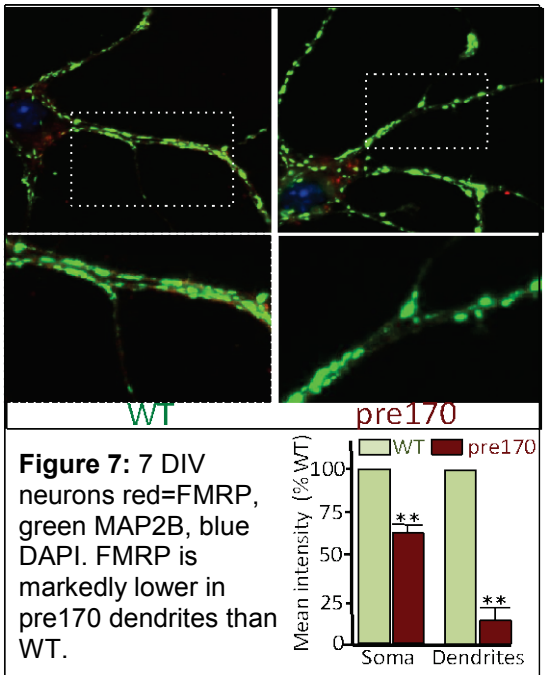


Figure 7: 7 DIV neurons red=FMRP, green MAP2B, blue DAPI. FMRP is markedly lower in pre170 dendrites than WT.

Task 4. ***Extension of small molecule and antagomiR targeting of the FMR1 mRNA in mouse and human iPSC and transdifferentiated neurons (timeframe, < 36 months; Hagerman and Pessah).***

Brief overview: These studies, which have not yet commenced, will utilize human and mouse hippocampal neurons, as well as iPSC-derived and transdifferentiated neurons, to determine to what extent we can recover a neurotypical phenotype through up-regulation of FMRP. These studies will focus more on the electrical aspects of neuronal function, and will exploit FLIPR and MEA studies to look at network activity.

KEY RESEARCH ACCOMPLISHMENTS

- Quantified the effects of interfering with the miRNA-3′UTR interactions for the *FMR1* gene; the best target blocker was one that resulted in an average increase of ~70% in the level of FMRP. An unexpected observation was that part of this increase is likely do to protection of the mRNA itself from degradation.
- Developed and implemented a much faster method for quantifying the effects of various agents on the level of FMRP, using a FRET-based assay that allows rapid quantification of endogenous FMRP.

- Completed subcloning of human fibroblasts from normal and *FMR1* premutation individuals with *FMR1* expansion repeats in the mid-, high- and full range.
- Successfully obtained iPSCs, and a pair of isogenic premutation iPSC-derived NPCs from a mosaic premutation patient.
- Identified similar functional impairments in Ca^{2+} dynamics occur in both mouse premutation neurons and human iPSC-derived NPCs.
- Identified evidence for mitochondrial dysfunction in mouse premutation neurons.
- Discovered that although FMRP is modestly (25-40%) reduced relative to wild type, mouse high-range premutation neurons have nearly 7-fold less FMRP in their distal dendrites.
- Preliminary results indicate that high-range premutation neurons in culture (and brain tissue) have increased levels of Cdk5, ATM and P-ATM compared to respective preparations from wild type.

REPORTABLE OUTCOMES

Reportable outcomes that have resulted from this research include:

Pretto DI, Kumar M, Cao Z, Cunningham CL, Durbin-Johnson B, Qi L, Berman R, Noctor SC, Hagerman RJ, Pessah IN, Tassone F. Reduced excitatory amino acid transporter 1 and metabotropic glutamate receptor 5 expression in the cerebellum of fragile X mental retardation gene 1 premutation carriers with fragile X-associated tremor/ataxia syndrome. Neurobiol Aging. 2014 May;35(5):1189-97.

CONCLUSION

Our fundamental observation is that the native FMRP levels can be increased through blocking certain microRNA-mRNA interactions, since the protein is important for brain development, and its loss is accompanied by both intellectual and behavioral disability. Our research should lead to the development of therapeutic agents that increase the cellular production of production of FMRP. Increasing the expression of FMRP holds the potential to correct ALL of the clinical domains of fragile X syndrome. Moreover, since posttraumatic stress disorder (PTSD) has been described in fragile X syndrome and in premutation carriers, the proposed studies may lead to treatments that reduce the PTSD risk as well.

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APPENDICES

N/A